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Identification of a Linear Neutralization Domain in the Protein VP2 of African Horse Sickness Virus

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Overlapping fragments of the outermost capsid protein VP2 of African horse sickness virus serotype 4 (AHSV-4) have been expressed in *Escherichia coli*. Horse sera from infected and vaccinated animals, rabbit sera, and mice monoclonal antibodies specific for AHSV were used to screen these fragments for antigenic regions. The screening revealed that the major antigenic domain of the AHSV-4 VP2 is localized in a central region (amino acids 200 to 413) and that both the N-terminal region (aa 1–159) and the half C-terminal region (aa 414–1060) are not immunogenic. All the fragments containing a region between amino acids 253 and 413 (fragment H) were able to elicit consistently high titers of neutralizing antibodies. The ability of several subfragments of this region to evoke neutralizing antibodies indicates the presence of several sites inside this domain. However, neutralizing antibodies in sera of horse infected or vaccinated with attenuated viruses were not absorbed by fragment H, indicating that this domain is not immunodominant in AHSV. This information might be useful in designing a subunit vaccine against AHSV infection.

INTRODUCTION

African horse sickness virus (AHSV) is a member of the orbivirus subgroup within the Reoviridae family (Holmes, 1991). It is enzootic in sub-Saharan Africa, although occasional outbreaks have occurred in northern Africa, the Middle East, and southern Europe (Lubroth, 1988; Rodríguez *et al.*, 1992; Mellor, 1993). The virus is propagated through the bite of gnats of the genus *Culicoides* (Du Toit, 1944), which constitute the natural reservoir of the virus. AHSV causes an acute disease in horses, with a high mortality rate. Nine different serotypes of AHSV have been identified (McIntosh, 1958; Howell, 1962). AHSV serotype 4 has been responsible for the most recent outbreaks in Spain and Portugal. The molecular characteristics of this serotype have been characterized thoroughly in the last years (see Roy *et al.*, 1994, for a review).

The orbivirus particles have 10 double-stranded RNA segments encapsidated by double capsids. The outermost layer is formed by two proteins, VP2 and VP5 (Bremer, 1976). AHSV capsid protein VP2 (124 kDa) elicits serotype-specific antibodies (Bremer *et al.*, 1990) and is the major target of the neutralizing response of the host (Ranz *et al.*, 1992; Burrage *et al.*, 1993; Martínez-Torrecuadrada *et al.*, 1994). From a collection of 53 anti-AHSV monoclonal antibodies (MAbs), 11 neutralizing MAbs were obtained, and all were specific for VP2 and not against other structural proteins of the viral capsid (Ranz *et al.*, 1992). However, although a baculovirus-derived recombinant VP2 was able to induce neutralizing antibodies, it was recognized very weakly by 9 of the 11 neutralizing MAbs (Martínez-Torrecuadrada, in preparation), indicating that most of the neutralization epitopes are likely to be discontinuous, as happens in bluetongue virus (BTV) (Rossitto and MacLachlan, 1992). The role of AHSV VP5 in neutralization remains unclarified.

To investigate and characterize the neutralizing activity in AHSV we have carried out an epitope mapping of VP2. To this end we have expressed overlapping fragments of AHSV-4 VP2 using a prokaryotic system (*Escherichia coli*), and we have used them to screen a collection of horse sera and MAbs. This system presents the limitation that, in general, only antibodies directed to linear B-cell epitopes will be detected. However, in certain cases linear B-cell epitopes are sufficient to induce neutralizing antibodies and to confer total protection to the target animal (Langeveld *et al.*, 1994).

In this report, we describe mapping of a linear neutralization domain on AHSV VP2. In addition, we demonstrate that some recombinant VP2 fragments are able to elicit neutralizing antibodies in mice and rabbits.

MATERIALS AND METHODS

Cells, viruses, and bacterial strains

A permanent line of Vero cells (ATCC CCL 81) was used for virus propagation and neutralization assays. Cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL) supplemented with 10% fetal calf serum (FCS) plus antibiotics and maintained at 37° in

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an 8% CO_2 atmosphere. AHSV particles were purified according to the procedure described previously (Martínez-Torrecuadrada *et al.*, 1994).

HMS174 and XL-1 Blue *E. coli* strains were used as the host for initial cloning of target DNA into the pET vectors (Studier and Moffat, 1986) and for maintaining plasmids. *E. coli* strain BL21 (DE3)pLysS (F^-, *ompT*$^-$, $r_B^- m_B^-$) (Grodberg and Dunn, 1988), kindly provided by M. A. de Pedro (Centro de Biología Molecular, Madrid, Spain), was used as bacterial host for expression. *E. coli* strain GM119, lacking the *dam* methylase (provided by J. Ayala, Centro de Biología Molecular, Madrid, Spain), was used to prepare plasmid DNA to be digested by restriction enzymes *Bcl*I and *Cla*I, which are methylation-sensitive.

Polyclonal and monoclonal antibodies

Horse sera from different sources, either from AHSV-4-infected horses or from horses vaccinated with live attenuated monovalent vaccine specific for AHSV-4, were used. Horse sera specific for the nine AHSV serotypes were kindly provided by J. M. Sanchez-Vizcaino (CISA–INIA, Madrid, Spain).

Nine MAbs which react with AHSV-4 VP2 by immunoblotting (8BF2, 8BC2, 8CB11, 8DB11, 8BB1, 8DA6, 8CE4, 8AE6, and 10BB4) and are nonneutralizing (Ranz *et al.*, 1992) were used to screen the overlapping fragments and the entire VP2. Rabbit serum anti-recombinant AHSV-4 VP2 was prepared as described previously by Martínez-Torrecuadrada *et al.* (1994).

Construction of different AHSV-4 VP2 expression plasmids

DNA manipulations were performed following standard cloning techniques (Sambrook *et al.*, 1989). The complete coding sequence of VP2 was obtained from the recombinant plasmid pUC4-AHSV-4.2 (Martínez-Torrecuadrada *et al.*, 1994). The strategy for the selection of digestions was based on the restriction pattern of the L2 gene of AHSV serotype 4 (Iwata *et al.*, 1992), as is shown in Fig. 1. The VP2 fragments generated were the following: VP2 gene; A (nt 12–2075, *Bam*HI fragment); B (nt 2075–3229, *Bam*HI fragment); C (nt 12–516, *Bam*HI to *Bcl*I); D (nt 606–2075, *Bcl*I to *Bam*HI); E (nt 12–1251, *Bam*HI to *Cla*I); F (nt 1251–1898, *Cla*I fragment); G (nt 439–770, *Hin*dIII fragment); H (nt 770–1251, *Hin*dIII to *Cla*I); I (nt 606–1251, *Bcl*I to *Cla*I); J (nt 606–730, *Bcl*I to *Sau*3AI); K (nt 730–860, *Sau*3AI fragment); L (nt 860–1149, *Sau*3AI fragment); and M (nt 1149–1251, *Sau*3AI to *Cla*I).

The fragments with *Bam*HI-compatible ends were ligated directly with *Bam*HI-digested phosphatase-treated pET3 (a, b, and c, depending on the reading frame), or pET3x (Studier *et al.*, 1990) in the cases of fragments F and M, since a larger fusion protein was required to confer more stability. Fragments E, F, G, and H, lacking *Bam*HI-compatible ends, were first subcloned in a pMTL plasmid in order to generate *Bam*HI-compatible ends.

The ligation mixtures were used to transform XL-1 Blue or HMS174 competent cells. The resulting colonies were screened by digestion with appropriate restriction enzymes. In order to check the orientation of the insert and the junction sequences, plasmids pET3 and pET3x containing the VP2 sequences were sequenced by the dideoxynucleotide method. The oligonucleotide primers used for the sequencing were 5′ CTTTAAGAAGGAGATATAC 3′ for pET3 and 5′ CTATCCGCAACGTTATGGGC 3′ in the case of pET3x. Competent cells of the BL21 (DE3)pLysS strain were transformed with all the recombinant pET3 plasmids. Chloramphenicol and ampicillin agar plates were used to select the transformed cells.

Growth, induction, and analysis of *E. coli*-transformed cells

Single clones of *E. coli* BL21 (DE3)pLysS cells containing the recombinant pET-derived plasmids were grown overnight at 37° in LB medium in the presence of 50 μg/ml ampicillin and 40 μg/ml chloramphenicol. Then, the cells were diluted 100-fold in LB medium plus antibiotics. Once the cultures reached an A_{600} of 0.8 they were induced by the addition of 0.4 m*M* isopropyl thiogalactopyranoside (IPTG) (Boehringer-Mannheim). Twenty-milliliter cell cultures were harvested 3 hr after induction by centrifugation at 4000 rpm for 5 min, washed twice with phosphate-buffered saline (PBS), and resuspended in a final volume of 500 μl of PBS. A volume of loading buffer (10 m*M* Tris–HCl, pH 6.9, 10% SDS, 10% β-mercaptoethanol, 0.02% bromophenol blue, 25% glycerol) was added to each sample and the mixture was heated at 100° for 5 min before analysis on a 7 to 15% SDS–polyacrylamide gradient gel (Laemmli, 1970). Detection of proteins was carried out by staining with Coomassie brilliant blue.

Immunoblotting analyses

Proteins resolved by SDS–PAGE were electroblotted to a Trans-Blot membrane (Bio-Rad). The membrane was incubated for 1 hr at room temperature in blocking buffer (3% skimmed milk–0.05% Tween 20 in PBS). After blocking, two types of antibodies were used, horse sera (infected or vaccinated horses) or monoclonal antibodies against AHSV-4 VP2. Where indicated, horse sera specific for nine AHSV-serotypes were used. To avoid unspecific reactions, the horse sera were preadsorbed with acetone powders of whole bacteria for 1 hr at 4° and then added to the membranes at a 1:250 dilution in blocking buffer. The filters were incubated at room temperature for more than 2 hr. After four 10-min washes with 0.05% Tween 20 in PBS, bound antibody was detected by peroxidase-conjugated anti-horse antibodies (Bio-Yeda, Israel) diluted at 1:500 in blocking buffer. 4-Chloro-1-

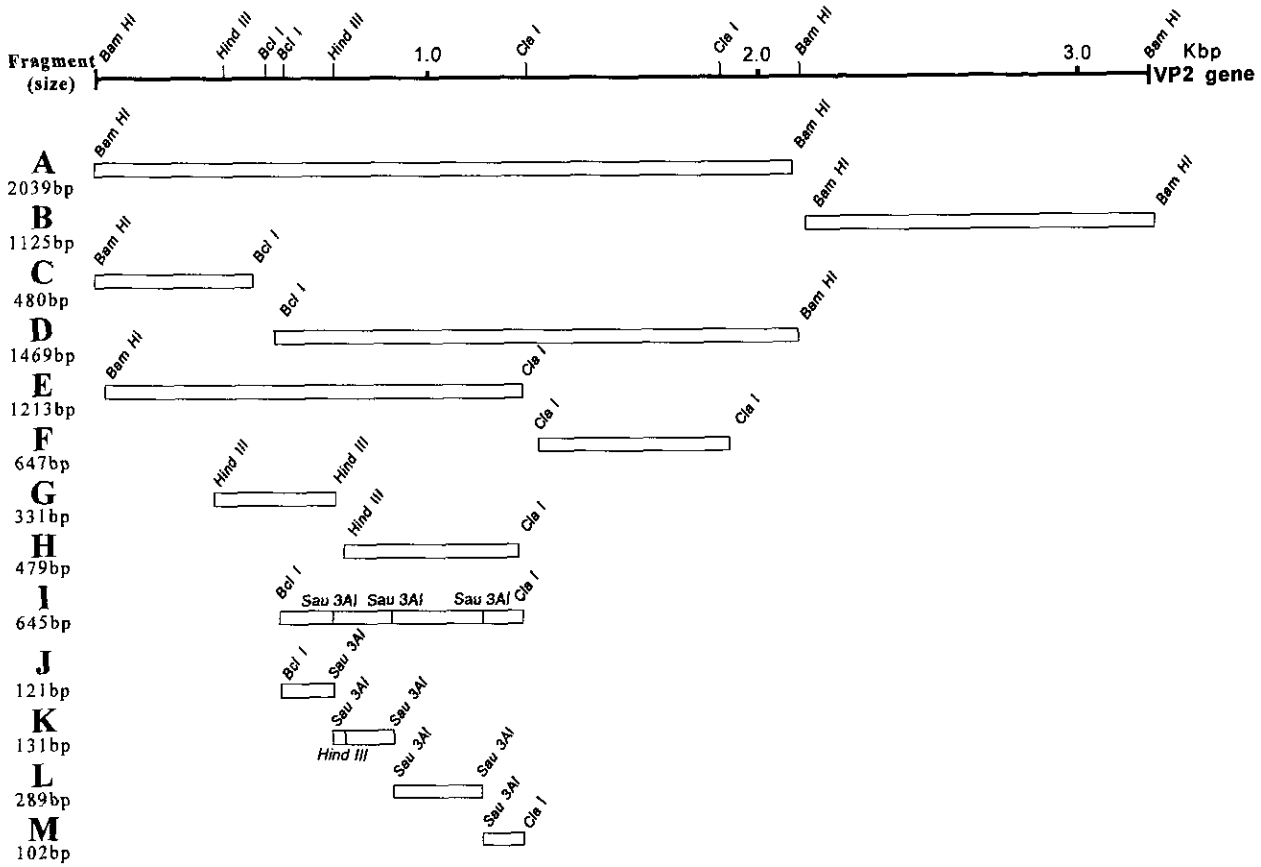

FIG. 1. Map of AHSV-4 VP2 fragments. VP2 fragments were constructed as described under Materials and Methods, using convenient restriction enzyme sites. The open reading frame of VP2 is depicted as a dark line with the restriction enzymes sites used in the fragmentation process. The closed boxes (from A to M) with the restriction enzymes sites at the ends represent the location and length of fragments that were generated by restriction enzyme digestions to be subsequently cloned in pET3 expression plasmids. The size of every fragment is indicated below the letter.

naphthol (0.5 mg/ml) (Sigma); 17% (v/v) methanol, and 0.015% hydrogen peroxide in PBS were used to detect attached conjugate. The reaction was stopped by rinsing the membranes with distilled water.

The bound monoclonal antibody was detected using an alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Sigma), nitroblue tetrazolium chloride (Gibco BRL), and bromochloroindoyl phosphate (Pierce) as substrates following the manufacturer's protocol.

Purification of recombinant proteins

After 3 hr of incubation with IPTG, the induced cells were collected by centrifugation and resuspended in 50 m*M* Tris–HCl, pH 8.0, and 2 m*M* EDTA. The bacteria were subjected to three freeze–thaw cycles and sonicated three times at 22 μm for 30 sec each in a Soniprep apparatus (MSE, UK). This treatment was enough to lyse the cells due to the presence of resident lysozyme codified by pLysS. The bacterial lysate was centrifuged for 15 min at 12,000 *g*, washed once with 50 m*M* Tris–HCl, pH 8.0, 10 m*M* EDTA, 0.5% Triton X-100, and 100 m*M* NaCl to remove soluble proteins, and washed three times with buffer A (50 m*M* Tris–HCl, pH 8.0, 0.5% glycerol, 1 *M* NaCl). The final pellet was then solubilized in 50 m*M* Tris–HCl, pH 8.0, 0.5% glycerol that contained 2 *M* guanidinium hydrochloride (buffer B) due to the insolubility of the expressed polypeptides. After centrifugation at 12,000 *g* for 15 min, the supernatant with the guanidinium chloride-soluble fraction, highly enriched in recombinant proteins, was dialyzed against buffer A and subsequently against PBS.

Immunization of animals

Groups of five 3-week-old BALB/c mice were immunized by intraperitoneal injection on Days 0, 15, and 45. Fifty micrograms of recombinant proteins, semipurified as described above, in 100 μl of PBS was mixed with the same volume of Freund's complete adjuvant (Sigma) for the first inoculation and with Freund's incomplete adjuvant (Sigma) for the subsequent boosters. Immunized mice were bled on Days 0, 60, and 80 and the sera were tested by enzyme-linked immunosorbent assay (ELISA) and neutralization tests.

Female New Zealand White rabbits, 7 weeks old, were immunized intramuscularly in the rear legs. Fifty micrograms of semipurified proteins in 0.5 ml of PBS was

mixed with the same volume of Freund's complete adjuvant. Every 2 weeks the rabbits received a booster immunization (at least two) with 50 μg of the semipurified fragments in Freund's incomplete adjuvant.

ELISAs

Two types of ELISAs were carried out: one for detection of VP2 fragment-specific antibodies and the other for virus-specific antibodies. Microtiter 96-well plates (Nunc, Denmark) were coated overnight at 4° with a dilution 1:100 of guanidinium chloride-soluble *E. coli*-derived VP2 fragments in 100 μl 0.05 *M* carbonate buffer, pH 9.6. Residual protein was removed by washing three times with PBS containing 0.02% Tween 20. Plates were blocked with 5% dry skimmed milk in PBS for 1 hr at 37° and incubated with twofold serial dilutions of corresponding horse serum in 5% milk–0.05% Tween 20–PBS for 1 hr at 37°. After washing, plates were incubated with peroxidase-labeled Protein G (Pierce, diluted 1:1000 in blocking solution) for 1 hr at 37°. Horseradish peroxidase activity was detected by adding ABTS (2,2′ azino-di[ethylbenzothiazoline]) (Sigma) as substrate. The reaction was stopped by the addition of 1% SDS and the optical density of the samples was determined at 405 nm in an ELISA reader (Bio-Tek Instruments, USA).

Detection of virus-specific antibodies in the mouse and rabbit sera raised against the different fragments was determined by indirect ELISA as described (Martínez-Torrecuadrada *et al.*, 1994).

Neutralization activity assays and absorption of this activity in sera

Neutralization was performed as described previously (Martínez-Torrecuadrada *et al.*, 1994). Briefly, a known amount of AHSV-4 (1.5×10^4 PFU/well) was incubated in a 96-well plate with 50 μl of serum at different dilutions for 2 hr at 37°. Then, samples were added to 1.5×10^4 Vero cells/well and after 4 days incubation, the cell monolayers were washed with PBS and stained with 1.5% crystal violet in 50% ethanol in water for 20 min. The level of protection was calculated by visual screening of the infected monolayers. The neutralizing titer was calculated as the reciprocal value of the highest serum dilution causing 50% reduction of cell monolayer. All incubations were carried out in the absence of serum complement.

To block neutralizing antibodies, the recombinant proteins (at concentrations from 0.1 to 0.01 mg/ml) were incubated for 2 hr at 37° with an equal volume of serum at different dilutions in DMEM containing 5% FCS. The mixtures were incubated with virus and processed as described above for the neutralization assay.

RESULTS

Cloning of different AHSV-4 VP2 gene products in pET expression vectors

The sequences covering the complete gene and various regions of the VP2 protein were excised from plasmid pUC4-AHSV-4.2 (Martínez-Torrecuadrada *et al.*, 1994), which contains the entire VP2 coding sequence (Fig. 1). Fourteen pET-derived recombinant plasmids were constructed containing overlapping fragments of the VP2 with various lengths from 0.1 to 2.0 kb, as shown in Fig. 1 and Table 1. They were designated pET3a-VP2, pET3a-ΔA, pET3c-ΔB, pET3a-ΔC, pET3b-ΔD, pET3a-ΔE, pET3xa-ΔF, pET3c-ΔG, pET3b-ΔH, pET3a-ΔI, pET3b-ΔJ, pET3a-ΔK, pET3c-ΔL, and pET3xb-ΔM. The expression vector pET3 was mainly used for this purpose, although pET3x was also chosen for those fragments too small to be expressed without larger fusion proteins, such as pET3xb-ΔM, or for toxic or unstable plasmids (pET3xa-ΔF). The plasmid pET3a-ΔA was also transformed into *E. coli* strain GM119, which is deficient in *dam* methylase, in order to provide nonmethylated DNA to be further digested by methylation-sensitive restriction enzymes such as *Bcl*I and *Cla*I, which were used to generate new subfragments. The correct reading frames and orientation of the inserts in the recombinant plasmids were verified by sequence analysis and restriction enzyme digestions, respectively.

Expression of fusion proteins in *E. coli*

Bacteria were harvested 3 hr after IPTG induction, sufficient time for substantial accumulation of target protein but before the culture could be overgrown with cells that had lost plasmid or were unproductive. The production in *E. coli* of fusion proteins containing AHSV-4 VP2 fragments was examined by Coomassie blue staining of SDS–PAGE gels (Fig. 2). A strong protein band was noticed in every construct, with a size in agreement with the molecular weight expected for every polypeptide. The fusion proteins showed some variations in expression levels (from 0.1 mg of fragment M to 0.02 mg of fragment B per ml of culture, as estimated by visual comparison with known quantities of BSA in SDS–PAGE analyses), although in every case it was sufficiently abundant to allow the purification. In general, VP2 fragments were expressed more efficiently when the fusion proteins were composed by a fusion of 260 aa rather than the 11 aa of the gene *10* protein. In all the cases, the expressed proteins were insoluble, although they could be easily solubilized with 2 *M* guanidinium hydrochloride. However, the proteins became insoluble again when the guanidinium chloride was removed from the preparations by dialysis, except fragments F and M, which remained soluble.

This treatment was used to enrich the content of re-

TABLE 1

Summary of VP2 Fragment Expression Constructs and Reactivity of AHSV-4-Specific Antibodies by Immunoblotting

Fragment								
Name	Size (bp)	Subcloning vectors[a]	pET3	pET3x	Resulting plasmid	Horse α-AHSV-4 sera	Rabbit α-VP2 sera[c]	MAbs
VP2	3160	—	a	—	pET3a-VP2	+	+	+
A	2039	—	a	—	pET3a-ΔA	+	+	+
B	1125	—	c	—	pET3c-ΔB	−	−	−
C	480	—	a	—	pET3a-ΔC	−	−	−
D	1469	—	b	—	pET3b-ΔD	+	+	+
E	1213	pMTL22	a	—	pET3a-ΔE	+	+	+
F	647	pMTL24	—	a	pET3xa-ΔF	−	+	−
G	331	pMTL22	c	—	pET3c-ΔG	−	−	−
H	479	pMTL22	b	—	pET3b-ΔH	+	+	+
I	643	—	a	—	pET3a-ΔI	+	+	+
J	121	—	b	—	pET3b-ΔJ	−	−	−
K	131	—	a	—	pET3a-ΔK	−	−	+[d]
L	289	—	c	—	pET3c-ΔL	+	+	+[e]
M	102	—	—	b	pET3xb-ΔM	+	+	−

[a] When it was needed.
[b] Pool of AHSV-4-infected and vaccinated horse sera.
[c] Sera raised against baculovirus-derived proteins.
[d] Fragment K was recognized by MAbs 8BB1, 8BC2, 8BF2, 8CE4, 8AE6, 8DB11, 8CB11, and 8DA6.
[e] MAb 10BB4 only reacted with fragment L.

combinant protein. The purity of the fusion proteins obtained after guanidinium–hydrochloride solubilization was >75% as assessed by Coomassie blue-stained SDS–PAGE gels. These partially purified fusion proteins were used to carry out the epitope mapping and to immunize mice and rabbits.

Reactivity of AHSV-4-specific antibodies with the fusion proteins

Epitope mapping was accomplished by probing the reactivity of different fragments of the VP2 gene with a collection of polyclonal horse antisera, rabbit serum anti-recombinant VP2, and MAbs. Two techniques were used: immunoblotting (Fig. 3) and ELISA (Table 2). The reactivity of all the sera with *E. coli*-derived VP2 fragments by immunoblotting is summarized in Table 1. The entire VP2 gene, expressed in baculovirus, was recognized by all sera used in this study. Therefore, initially VP2 was fragmented into two halves. When these fragments were examined, the C-terminal fragment B was not recognized by any monoclonal or polyclonal antibodies, indicating a low antigenicity. However, since the N-terminal fragment A was recognized by the polyclonal and MAbs, it was divided in four portions using *Bcl*I and *Cla*I sites. Expression of *Bcl*I fragments yielded two polypeptides, C and D, of 18.6 and 54.3 kDa, respectively. *Cla*I digestions generated two other proteins, E and F, of 45.8 and 24 kDa (Fig. 1). Fragments D and E were well recognized by immunoblotting by all the tested sera (Table 1). In contrast, neither serum gave a positive reaction with N-terminal fragment C nor with fragment F (Table 1, Fig. 3). These results indicated that the main antigenic determinants are located in a region between nucleotides 606 and 1251 (VP2 residues 200 to 413).

To confirm this finding, a fusion protein encoding that region was constructed (fragment I) using the region between *Bcl*I and *Cla*I sites at nt 606 and 1251, respectively. As expected, fragment I was recognized by all polyclonal and MAbs tested.

To define the antigenic domains more precisely, fragment I was further divided in two groups of segments: G and H through the *Hin*dIII site present at nt 770, and fragments J, K, L, and M using the three *Sau*3AI sites of fragment I. Fragment H was recognized by all polyclonal horse sera (Fig. 3) and MAbs (Table 1). For the small polypeptides (J, K, L, and M), different reactions were observed. The horse sera, but not MAbs, reacted preferentially with fragment M (aa sequence: PNKGKWKEHIKEVTEKLKKAQTENGGQPCQVSI) and slightly with fragment L. Fragment K was recognized by all the MAbs except 10BB4 (data not shown) and not by the polyclonal sera as shown in Fig. 3. The amino acid sequence of this domain is SAMYSGKGPLNDLRVKIERDDLSRETIIQ. MAb 10BB4 reacted with fragment L and not with the other small fragments, providing evidence for the location of the 10BB4 epitope in fragment L. No epitopes were found in fragment J.

To confirm the immunoreactivity of the VP2 fragments, each fragment was used for the detection of specific antibodies in horse and rabbit sera by ELISA (Table 2).

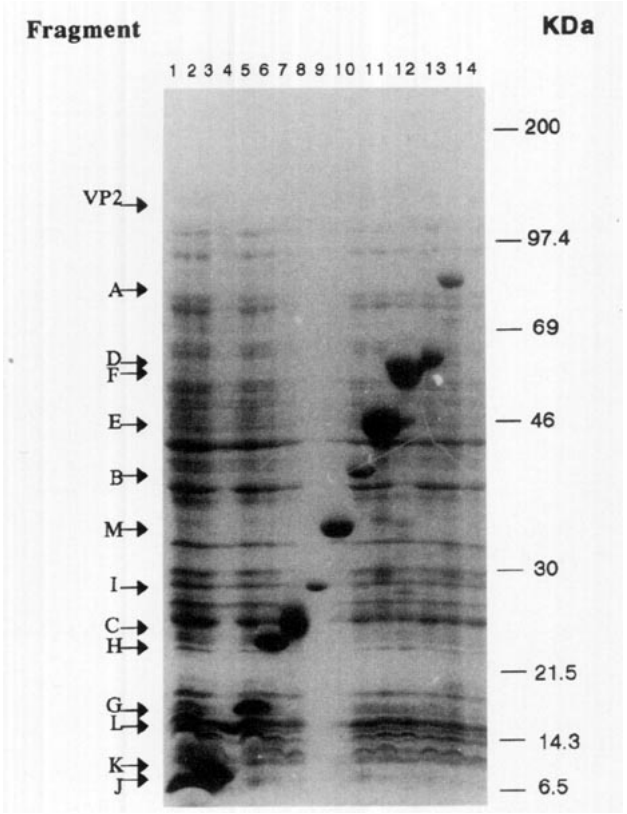

FIG. 2. Expression of the AHSV-4 VP2 fragments in *E. coli* (BL21(DE3)-pLysS strain). Bacteria containing the recombinant pET3 expression plasmids were induced during 3 hr with 0.4 m*M* IPTG. After extraction in protein dissociation buffer, proteins were fractionated on a 7 to 15% SDS–polyacrilamide gradient gel and stained with Coomassie blue. Each lane represents cell lysates transformed with lane 1, pET3b-ΔJ; lane 2, pET3a-ΔK; lane 3, pET3c-ΔL; lane 4, pET3c-ΔG; lane 5, pET3b-ΔH; lane 6, pET3a-ΔC; lane 7, pET3a-ΔI; lane 8, pET3xb-ΔM; lane 9, pET3c-ΔB; lane 10, pET3a-ΔE; lane 11, pET3xa-ΔF; lane 12, pET3b-ΔD; lane 13, pET3a-ΔA; and lane 14, pET3a-VP2. Molecular weight marker sizes are given in kDa and positions of every fusion protein are also indicated.

These ELISA data correlated well with those obtained by immunoblotting. As expected, all AHSV-specific horse antisera showed a high reactivity against fragments D, H, I, and L. However, those fragments containing either the N-terminus (fragments A, C, and E) or the C-terminal half of VP2 (B and F) reacted poorly, if at all, with sera by ELISA. Antibodies against fragments G, J, K, and M could be detected in some horse sera, but at a low level. The major difference between rabbit and horse sera is the strong recognition of fragments A, E, and M by rabbit sera. Probably these fragments contain epitopes not exposed on the surface. However, fragments B, C, G, J, and K did not react at all with the rabbit anti-recombinant VP2 serum.

AHSV-4 neutralization by antibodies elicited by expressed VP2 fragments

To assign a biological function to the reacting antibodies, all of the VP2-derived fragments were used for injection into mice and to evaluate their ability to elicit neutralizing antibodies. As expected, all mice developed antibodies to the immunizing fragments and the antisera reacted with the whole virion by indirect ELISA (data not shown).

The mice sera were assayed for neutralizing activity (Table 3). Several fragments (D, E, H, I, L, and M) elicited neutralizing antisera. Fragment H was especially effective in inducing neutralizing activity in mice and in rabbits, with titers of 640 comparable to those present in sera from naturally AHSV-4-infected or vaccinated horses. Fragments L and M, which are contained inside H, also elicited a high neutralizing titer (320 and 80, respectively). This result suggests that a number of epitopes responsible for viral neutralization are located within fragment H. In contrast, fragments without the sequence of fragment H did not evoke neutralizing antibodies, although they had the capability to elicit antibodies against AHSV virions, as demonstrated by ELISA.

Reactivity of fragment H with specific antisera for the nine AHSV serotypes

Since there are nine AHSV serotypes, fragment H was used to determine the significance of this neu-

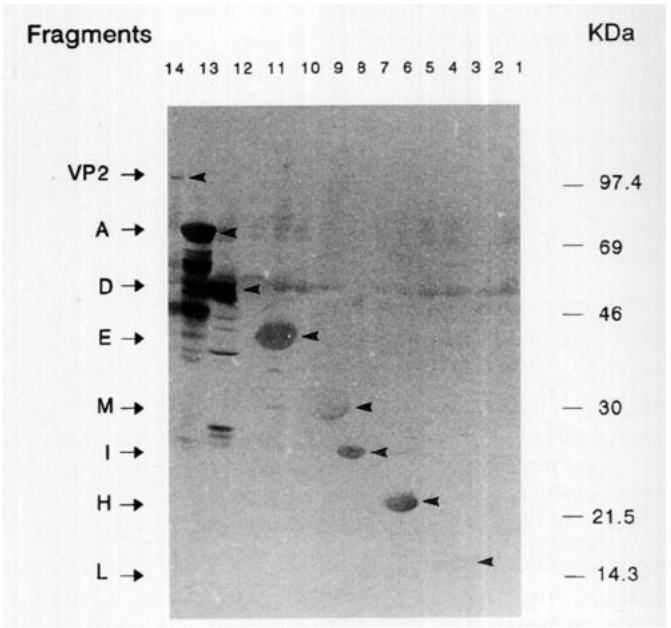

FIG. 3. Western blot analysis showing the reactivity of AHSV-4 VP2 fragments. Cell lysates overexpressing each fragment were prepared, mixed with protein sample buffer, and resolved on a 7 to 15% SDS–polyacrylamide gradient gel. Separated proteins were transferred to nitrocellulose membranes and the filters were incubated with a pool of AHSV-4-infected and vaccinated horse sera. Reactions were detected using HRP-conjugated anti-horse antibodies (see Materials and Methods). The lane numbers at the top indicate the VP2–fragment fusion proteins. Lane 1, J; lane 2, K; lane 3, L; lane 4, G; lane 5, H; lane 6, C; lane 7, I; lane 8, M; lane 9, B; lane 10, E; lane 11, F; lane 12, D; lane 13, A; and lane 14, VP2. Only those fragments giving a positive reaction are indicated.

TABLE 2

Immunoreactivity Against AHSV-4 VP2 Fragments Determined by ELISA

Serum	Fragment												
	A	B	C	D	E	F	G	H	I	J	K	L	M
Horse 173	+	−	−	++++	+	−	−	+++	++++	−	+	++++	+
Horse 187	+	−	−	++	−	−	+	++	++	+	−	++	−
Horse 321	+	−	−	++++	−	−	+	++	++	+	−	++	+
Horse 15.15	+	−	+	++++	−	−	+	++++	++++	+	++	++++	+
Horse control	−	−	−	−	−	−	−	−	−	−	−	−	−
Rabbit anti-VP2	+++	−	−	++++	++++	++	−	++++	++++	−	−	++++	+++
Rabbit control	−	−	−	−	−	−	−	−	−	−	−	−	−

Note. Anti-baculovirus-expressed VP2 rabbit serum and sera from horses vaccinated with monovalent vaccine (187 and 321) or naturally infected with AHSV-4 (173 and 15.15) were screened by ELISA for antibodis against AHSV-4 VP2 fragments at a 1:100 dilution of sera. The strength of the reaction was expressed according to optical density values at 405 nm (−, OD is below cutoff; +, OD is between cut off value and 0.4; ++, OD = 0.4−0.6; +++, OD = 0.6−1.0; ++++, OD > 1.0).

tralizing domain in the other eight AHSV serotypes. To this end, fragment H was probed in an immunoblotting assay with each prototype antisera against the eight heterologous AHSV serotypes. As shown in Fig. 4, the results indicated that sera from serotypes 6, 7, and 8 were able to recognize fragment H from serotype 4. However, the prototype sera for serotypes 1, 2, 3, 5, and 9 did not react with it. This result indicates the conservation of this domain in four different serotypes of AHSV, despite the high variability of the VP2, widening the potential interest of this fragment for the development of a subunit vaccine.

TABLE 3

Neutralizing Activity in Mice and Rabbit Sera Elicited by AHSV-4 VP2 Fragments

Serum	Neutralization titer[a]
Mice control	<5
Mice anti-fragment A	<5
Mice anti-fragment B	<5
Mice anti-fragment C	<5
Mice anti-fragment D	160
Mice anti-fragment E	80
Mice anti-fragment F	<5
Mice anti-fragment G	<5
Mice anti-fragment H	640
Mice anti-fragment I	160
Mice anti-fragment J	<5
Mice anti-fragment K	<5
Mice anti-fragment L	320
Mice anti-fragment M	80
Mice anti-AHSV-4	160
Rabbit control	<5
Rabbit anti-fragment H	1280

[a] Neutralization titers represent the reciprocal of the highest dilution of the antiserum that gave 50% reduction in the number of plaques obtained in controls (preimmune rabbit and mouse sera) by Vero monolayer protection assay. A mice serum anti-AHSV-4 was used as positive control. Sera were inactivated for 30 min at 56° prior to use to remove complement.

Blocking of AHSV-4-neutralizing antibodies

To investigate the immunodominance of the neutralization domain within fragment H, a collection of 12 AHSV-4-neutralizing horse sera was tested in a Vero monolayer protection assay after absorption with fragment H (Table 4). The mouse anti-fragment H and rabbit anti-baculovirus-derived VP2 sera were used as controls and they were completely absorbed by fragment H at a concentration of 0.1 mg/ml. However, the neutralizing activity of infected horse sera was not inhibited. This result indicates that although fragment H is recognized by all the tested horse sera and it is able to induce neutralizing antibodies, its neutralizing domain is not immunodominant in in-

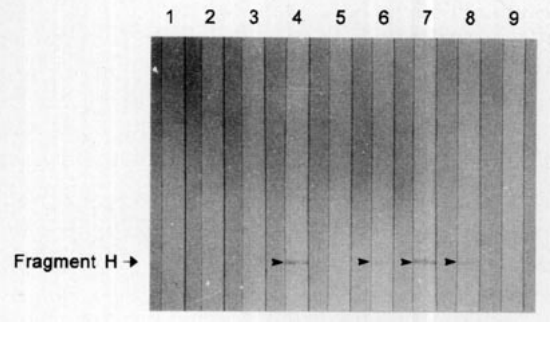

FIG. 4. Conservation of fragment H between the different AHSV serotypes. Semipurified fragment H was electrophoresed on a 12% SDS–polyacrylamide gel, and separated proteins were transferred to nitrocellulose filters for Western -blot analysis. The membranes were incubated with the prototype horse antisera against the different AHSV serotypes. The number of each serotype is indicated at the top of the membrane. Reactions were detected using peroxidase-labeled anti-horse IgG antibody and 4-chloro-1-naphthol as substrate. Arrowheads indicate the position of fragment H in those serotypes giving a positive reaction.

TABLE 4

Absorption of Neutralizing Activity by Fragment H

Serum	Neutralization titer[a]		
		Protein used for absorption	
	Medium	Fragment H (0.1 mg/ml)	Fragment H (0.01 mg/ml)
Mice α-fragment H	640	<10	40
Rabbit α-VP2	320	<10	20
Rabbit α-AHSV-4	640	640	640
Horse 28	2560	2560	2560
Horse 83	320	320	320
Horse 173*	1280	1280	1280
Horse 174*	2560	2560	2560
Horse 179	640	640	640
Horse 184	1280	1280	1280
Horse 187	640	640	640
Horse 201	2560	2560	2560
Horse 321*	1280	1280	1280
Horse 370	1280	1280	1280
Horse 6.15*	2560	2560	2560
Horse 15.15*	2560	2560	2560
Mouse preα-fragment H	<10	<10	<10
Rabbit preα-VP2	<10	<10	<10
Horse preα-AHSV-4	<10	<10	<10

[a] Neutralization titers were calculated as described under Materials and Methods. Different dilutions of sera were absorbed with an equal volume of *E. coli*-derived fragment H of the indicated concentration. The serum–fragment H mixture was introduced into the neutralization assay. Sera indicated as (*) were obtained from infected horses, and the remaining sera from vaccinated horses. Sera from preimmune rabbit, horse, and mouse were included as controls. Sera were inactivated for 30 min at 56° before using to remove complement.

fected horses and other conformational epitopes must play an important role in AHSV neutralization.

DISCUSSION

Recently, we have shown that a recombinant VP2 expressed in insect cells is able to induce neutralizing antibodies *in vitro* (Martínez-Torrecuadrada *et al.*, 1994), which suggests that VP2 may be useful as a subunit vaccine. However, to date the molecular characterization of the neutralization epitopes in AHSV VP2 has not been investigated. In orbiviruses, most of the epitope mapping has been carried out in BTV VP2 using monoclonal antibody-resistant mutants (Gould *et al.*, 1988, Gould and Eaton, 1990; DeMaula *et al.*, 1993) and synthetic peptides (Hwang and Li, 1993). In this study, a different approach was followed for AHSV. A collection of overlapping AHSV-4 VP2 fragments was expressed in *E. coli* to be probed with a collection of horse antisera and MAbs. We have shown in this report that the lowest antigenicity and immunogenicity of AHSV VP2 corresponds to the C-terminal half of the molecule, which was neither recognized by any sera or MAb nor elicited AHSV-specific antibodies. These data are in agreement with the computer analysis predictions, which show a high hydrophobicity for this region (data not shown). In general, the C-terminus is the most conserved region of the orbivirus VP2 sequences, implying a possible role in the interaction with other capsid proteins (i.e., VP5 and VP7) that makes this region not accesible to the surface of the capsid (Roy *et al.*, 1994). The VP2 N-terminal region was recognized by some horse antisera, but did not appear to have a strong immunogenicity. However, the highest antigenicity values were obtained with a central region, representing residues 200–413, and fragments within this domain.

Interestingly, several AHSV VP2 fragments were able to elicit neutralizing antibodies. In particular, all those proteins containing fragment H (aa 285 to 413) were capable of consistently eliciting neutralizing antibodies in mice and rabbits. This domain is reminiscent of the situation in BTV, where neutralization epitopes have been located in positions 328–335 (Gould and Eaton 1990) and 327–402 (DeMaula *et al.*, 1993). The common location of these neutralization domains in AHSV as in BTV suggests that these regions may be conserved because they are responsible for important functions (e.g., cell binding). In contrast, other neutralizing regions (residues 642–651) of BTV VP2 (Hwang and Li, 1993) were not identified in AHSV4 VP2. In this case, the predicted secondary structure is also different and it is not so hydrophilic in AHSV as in BTV.

Subdivision of fragment H into smaller fragments, which are also able to elicit neutralizing antibodies, indicates the presence of several sites within this large antigenic domain. The recognition patterns of these small fragments were different for horse sera and MAbs. Horse sera recognize fragment M, which elicits neutralizing antibodies. However, MAbs recognize fragment K, which does not. These data indicate the necessity of using sera from animals naturally infected for identifying epitopes, since the recognition pattern varies and is influenced by the process of immunization and the type of antigen used.

The neutralizing activity of the horse sera tested was not absorbed by fragment H, suggesting that this neutralizing site is not immunodominant in AHSV natural infection and probably is not exposed at the surface of the virion. This epitope might become active only after fragmentation, depolymerization, or denaturation of the antigen. In any case, the fact that antibodies have been obtained with consistently high neutralizing titers in all the animals described here (mice and rabbits) and their conservation through four different serotypes of AHSV makes these polypeptides promising candidates to be tested as vaccines in horses. In fact, linear epitopes play an important role in eliciting neutralizing antibodies that can be protective, as demonstrated in CPV (Langeveld *et al.*, 1994) or the V3 loop on the envelope surface pro-

tein of HIV-1 (Goudsmit *et al.,* 1988) and FIV (De Ronde *et al.,* 1994). This possibility is more attractive as no successful baculovirus-expression of AHSV-4 VP3 has been obtained yet, which makes ineffective for AHSV the BTV approach based on the synthesis of CLPs and VLPs (Roy *et al.,* 1992). The use of *E. coli*-derived products may present some advantages over baculovirus-expressed VLPs in terms of cost-effective production of large quantities of recombinant protein, rapid processing, and simplicity to incorporate new sequences corresponding to the different serotypes. This approach could also be used for other orbiviruses, as similar neutralization domains have been described in BTV.

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